THE JOURNAL OF INVESTIGATIVE DERMATOLOGY
Vol. 49, No. 2

Printed in U.S.A.

INFLUENCE OF STEROIDS ON FIBROBLASTS. I. AN *IN VITRO* FIBROBLAST ASSAY FOR CORTICOSTEROIDS*

DAVID L. BERLINER, M.D.† AND ANN G. RUHMANN, M.D.

INTRODUCTION

That corticosteroids act on a large number of cells *in vivo* and *in vitro* is a well recognized phenomenon (1–5). Lymphocyte control, stress reactions, inflammatory responses, and myriad other systemic functions are also affected crucially by naturally occurring and synthetic steroids (6–9). As there are many target cells for corticosteroid activity, so also have there been many systems devised for ascertaining the potency of these compounds. Correlations between the test systems and the various physiological functions they are measuring differ widely according to the *in vivo* organ and the *in vitro* technique employed. A high degree of correlation from an *in vitro* system to a physiological one is most desirable, not only because the ease and practicality of an *in vitro* assay would simplify testing of compounds destined for *in vivo* actions, but also because the accessibility and isolation of the *in vitro* system would make investigation of the basic mechanisms involved a simpler and faster matter. This is especially true in a system sensitive to fractions of micrograms per milliliter of fluid, which closely mimics physiological conditions.

The anti-inflammatory activity of corticosteroids is one of the important functions of that group of compounds (1, 5, 10). Connective tissues respond to inflammatory stimuli with a series of reactions which are inhibited by corticosteroids. At the cellular level, the fibroblast is intimately concerned with the process of inflammation, both as a manufacturer of connective tissue components and as the cell of origin or transformation for many other cell types (10, 11). The fibroblast can be grown, isolated from other connective tissue components, as a monolayer on glass or as a cell suspension. Various *in vivo* functions of the fibroblast are preserved and can be stimulated to action *in vitro* (11, 12) by the same substances that act on it *in vivo*. There is even a diurnal variation in numbers of connective tissue fibroblasts that parallels the diurnal differences in steroid output by the organism (13). Therefore, it is not surprising that fibroblasts react to steroids with morphological changes that are similar *in vivo* and *in vitro* (4, 10, 14, 15, 16), and that these reactions are correlated with inflammatory effects.

It is the purpose of this paper to elucidate in detail a method for studying, *in vitro*, the effects on fibroblasts of minute amounts of corticosteroids (from 0.0001 μg/ml to 10 μg/ml).

MATERIALS AND METHODS

Fibroblasts of clone 929, strain L, were grown as monolayers in Eagle's minimum essential medium (17) (Earle's balanced salt solution) containing 2 mM glutamine, 10% calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin sulfate. On day zero of the experimental period, 100,000 cells were inoculated into roller tubes 18 × 150 mm, which were filled with 5% CO_2 in air, and sealed with silicon rubber stoppers. Following a 24 hour stationary period to allow cellular attachment to glass, the tubes were rotated at ⅕ revolution per minute for an additional 4 days. Media were renewed on day 4, and cell counts performed on day 5. Removal of cells from glass surfaces was accomplished by thorough scraping with a rubber policeman, after the old growth medium had been removed and 5 ml of fresh medium added. Suspensions were vigorously pipetted 10 times to minimize cell clumping.

Conditions for counting cells were standardized. All tubes for one experiment were seeded from the same reservoir, after cell population was established by duplicate counts. A volume of 1.0 ml of a 100,000 cells/ml suspension was used to seed each tube.

Counts on cells subjected to experimental conditions were performed in an electronic cell

Received for publication Oct. 19, 1966.
This study was supported by USPHS Grant AM 09216-02.
Steroids were supplied by Merck, Sharpe and Dohme Research Laboratories, Lederle Laboratories, Squibb Laboratory and Syntex Corporation.
* Department of Anatomy, University of Utah Medical Center, Salt Lake City.
† Career Research Development Awardee, USPHS Grant 1-K6-CA-22,571-03.

counter.* The contents of each tube were diluted to 20 ml with medium that was first analyzed for background activity. Counts were conducted at machine settings which gave the best spread and amplitude on size distribution plot, with windows between 20 threshold units and infinity. The former corresponded to the low point in the trough separating cellular debris from intact cells. Each tube was counted twice to insure that there was no block in the aperture tube of the counter nor some other mechanical interference.

Substances whose effect on fibroblast growth were to be tested, were dissolved in culture medium and added to the cells at the time of original subculture. Steroids were weighed on a precision balance, and dissolved in absolute methanol to give appropriate concentrations (100, 1, or 0.01 μg/ml). Aliguots of these solutions were pipetted into 8 ounce prescription bottles to which a propylene glycol-methanol mixture had been added in such quantity as to give a final concentration of 1 μL propylene glycol/ml of finished medium. The methanol was then volatilized under a nitrogen stream in a water bath maintained at less than 50° C, until only steroid dissolved in propylene glycol remained. These bottles then received 100 ml each of fresh complete culture medium. Control tubes contained only propylene glycol in the medium.

RESULTS

Statistical Analysis

A preliminary study was performed to determine optimal seeding density and volume for experiments. A number of tubes were inoculated with 100,000 cells in 0.2 ml from a 1 ml pipette and in 1 and 5 ml volumes from 5 ml and 10 ml pipettes. Standard deviations of cell counts on day 5 after seeding were consistently lower among tubes seeded with 1 ml (S.D. 4–7%) and 5 ml (S.D. 1–2%) than among those seeded with 0.2 ml (S.D. 9–20%). Time considerations made the 5 ml inocula impractical. Therefore, 1.0 ml was chosen as the inoculum volume from all experiments. It was known from previous week-long experiments that a good growth of cells with consistent and optimal sensitivity to steroids could be obtained if 100,000 cells were allowed to grow for 5 days in roller tubes (4).

As was mentioned earlier, two counts were performed on each cell sample. Duplication on the counter was very good, and standard deviations between two counts on the same sample were always $\leqslant 1$.

To simplify calculations, both counts on each tube were averaged, this value being used as the true count for that sample. Repeated determinations with the same sample indicated that the counts followed a Poisson distribution, and based on Poisson statistics, it was determined that an error less than 2% could be attained with 95% confidence by counting a total of 9600 cells per tube.

Let L be a sample count and S_L the variance of that count (18):

$$\begin{aligned} .02\,L &\geq 1.96\,(S_L) \\ .02\,L &\geq 1.96\,\sqrt{L} \\ \sqrt{L} &\geq 98 \\ L &\geq 9600 \end{aligned}$$

All counts were carried out to this level. Moreover, the 99% confidence level could be attained by counting 16,000 cells, and in the vast majority of cases, this level was reached. Counts over 50,000 were avoided because they necessitated progressively larger machine coincidence corrections.

Statistical analyses of the results obtained by counting five tubes per treatment group were performed by the analysis of variance method (19, 20). Since the raw data did not follow a normal distribution, logarithmic transformations were employed to achieve this end. Table I demonstrates the log-transformed data on a typical experiment involving cells treated with cortisol (4-pregnen 11β,17α,21-triol,3,20-dione) and fluocinolone acetonide (6α,9α-difluoro-11β, 16α, 17α, 21-tetrahydroxy pregnan-1-4-diene 3,20 dione 16,17-acetonide). Bartlett's Test (20) was applied to determine homogeneity of variances (S^2), a prerequisite for further statistical evaluation by the method of analysis of variance. As pictured in Table II, the analysis of variance was used to determine whether there was a difference between treatments, significant regression, and no deviation from parallelism. If these criteria were met, the regression coefficient (b) was determined and a fitted line constructed on the appropriate graph (see Fig. 1). (The criterion for curvature was not strictly observed, as in this case, where there is significant curvature, with $P < .01$.) This characteristic is not crucial for the statistical method. In the example, giving cortisol an assigned potency of 1:

$$b_{yx}\ (\text{cortisol}) = -0.2999$$
$$b_{yx}\ (\text{fluocinolone}) = -\ 0.2550$$

* Coulter Model B.

TABLE I

Example of raw data for a typical experiment

Logarithmic transformations of numbers of cells (divided by 10^4) per tube, sum of cells per group (ΣY) and variance per group (S^2).

Control	Cortisol (μg/ml)			Fluocinolone acetonide (μg/ml)			
	0.01	0.1	1.0	0.0001	0.001	0.01	0.1
1.974	1.987	1.796	1.438	1.776	1.637	1.239	1.124
1.810	1.897	1.698	1.296	1.812	1.495	1.231	1.006
1.784	2.042	1.727	1.305	1.848	1.459	1.259	0.937
1.837	1.912	1.767	1.352	1.695	1.515	1.197	1.064
1.885	1.869	1.768	1.308	1.845	1.516	1.178	1.101
ΣY9.290	9.698	8.756	6.699	8.976	7.622	6.104	5.232
$S^2$0.0056	0.0049	0.0015	0.0035	0.0040	0.0045	0.0011	0.0057

TABLE II

Summary of pertinent data for analysis of variance of a typical experiment

Source of Variation	*d.f.*	Sum of squares	Mean square	*F*	*P*
Total	39	3.890			
Within groups	32	0.123	0.0038		
Between groups	7	3.767	0.538	140.06	***
Control vs. treatment	1	0.509	0.509	132.59	***
Within treatments	6	3.257	0.543	141.31	***
Regression	1	2.511	2.511	653.47	***
Deviation from parallelism	1	0.0144	0.0144	3.75	—
Preparation	1	0.673	0.673	175.12	***
Curvature	3	0.0596	0.0199	5.17	**

*** < .001
** .01 – .001

Combined $b_c = -0.2678$
Index of significance $g = 0.00588$
Index of precision $\lambda = 0.232$
Potency ratio = 351.2
Fiducial limits potency ratio
= 234.6-525.7

The visualization of these data is seen in Figure 1. At least three concentrations of cortisol were included in each experiment to give continuity between separate experiments.

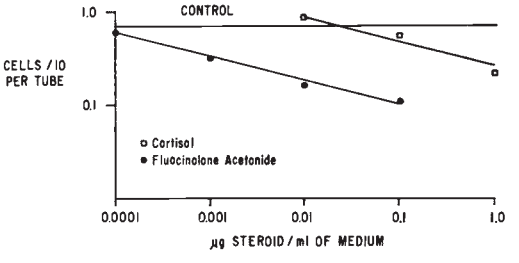

FIG. 1. Log-log plot of fibroblast growth under the influence of cortisol and fluocinolone acetonide. The lines are statistically fitted, with dose of steroid on the abscissa and millions of cells on the ordinate.

Propylene Glycol

Earlier studies of growth curves conducted over a 7-day period, established the lack of fibroblast inhibition or stimulation by propylene glycol at 1 μL/ml of medium, the concentration used in all experiments (4, 6). Results of more recent dose-effect studies conducted at the fifth day of culture growth, were subjected to analysis of variance and the Hartley multiple range tests (20, 21). The latter indicated there was no effect on fibroblast growth by 0.1, 0.2, 0.5, 1.0, or 2.0 μg of propylene glycol/ml of medium (Fig. 2). Growth was significantly depressed by all concentrations of propylene glycol above 5 μg/ml.

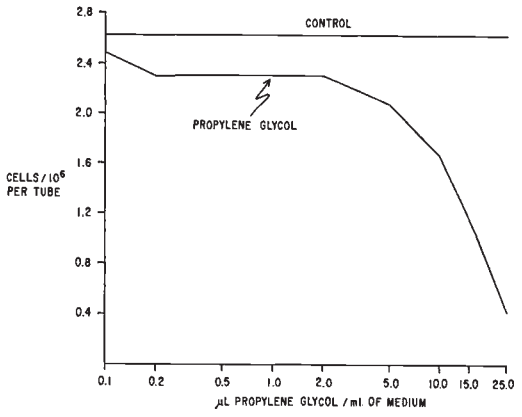

FIG. 2. Semilogarithmic plot of fibroblast growth under the influence of propylene glycol. Number of cells on the ordinate are plotted against propylene glycol concentration on the abscissa.

TABLE III

Summary of relative potencies and pertinent statistical data on several corticosteroids tested

Steroid	Potency ratio	Range	Indices of precision (λ)
Cortisone*			
Corticosterone	0.48	0.31–0.65	0.2
Cortisol	1.0	—	—
Prednisolone	1.7	1.1–2.3	0.2
Dexamethasone	7.5	6.9–8.0	0.1
Paramethasone	11.3	5.4–18.6	0.07
Triamcinolone acetonide	156	146–167	0.2
Fluocinolone acetonide	440	350–509	0.1

Range = lowest and highest determinations obtained from separate experiments.

λ = all modes of precision in systemic experiments equal to or less than values given.

* Cortisone as well as other 11-ketocorticosteroids have no effect in the fibroblast assay method (6).

Relative Potencies

Table III summarizes the relative potencies of a number of naturally occurring and synthetic steroids. The compounds are listed in order of increasing activity as fibroblast inhibitors. When cortisol, the most potent naturally occurring anti-inflammatory corticosteroid, is assigned a potency of 1, other steroids can be numerically compared to it. Cortisone is a growth stimulant in the fibroblast assay (6), and because growth under its influence shows no regression, a relative potency cannot be assigned to it. The fact that cortisone does occasionally exhibit anti-inflammatory action *in vivo* is explained by its biotransformation to cortisol (21, 22) which is minimal in this isolated tissue culture system (6). In the second paper of this series the relationship between this assay system and others will be discussed.

CONCLUSIONS

The finding that the behavior of a pure cell line in an isolated system is analogous in some respects to its behavior *in vivo* is a useful one. The method presented here is a simple and accurate means to elucidate structure activity relationships, steroid potencies, and cell metabolic behavior (4, 6). As an assay system, it is an excellent tool for analyzing corticosteroid effects by regression analysis. The index of precision ($\lambda = 0.232$ in the example given) is generally between 0.1 and 0.3, which is indicative of a consistently precise bioassay system. Systems giving variances small enough to produce precision indices below 0.1 are extremely rare, and require sample sizes greatly in excess of the five utilized in this system. The remarkable reproducibility of data in the tissue culture fibroblast assay is reflected in repetitions of potency ratios and consistency of dose-effect curves. Despite this good reproducibility, it must still be remembered that the assay is dependent on the tissue culture system, and therefore all the attendant possibilities for contamination (bacteria, virus, foreign cell line), altered growth due to changes in media lots, and spontaneous mutations. Structure-activity relationships have been shown to hold constant in this system in demonstrating the importance of the 11β- and 17α-hydroxyl groups, 16,17-acetonide structure, and 6α- and 9α-fluorine substitutions (4). Its anti-inflammatory assay potential is well illustrated in Table III.

The rank order of potency for topical anti-inflammatory activity of steroids increases from cortisol, through prednisolone (1,4 pregnadiene 11β,17α,21-triol,3,20-dione), dexamethasone (9α-fluoro 16α-methyl-11β,17α,21-trihydroxy

pregna-1,4-diene-3,20-dione), paramethasone (6α-fluoro 16α-methyl 11β,17α,21-trihydroxy pregnan-1,4-diene 3,20-dione), and triamcinolone acetonide (9α-fluoro-11β,16α,17α,21-tetrahydroxy-pregnan-1,4-diene 3,20-dione 16,17-acetonide), to the highly active compound fluocinolone acetonide (5, 23, 24). This order finds a direct parallel in the ranking of steroid activity by the fibroblast assay method.

An assay method, to be effective, must function in the physiological range. The fibroblast assay method is sensitive to a concentration of fluocinolone acetonide of 0.0005 μg/ml (approximately 10^{-9} M), and a concentration of cortisol of 0.1 μg/ml (approximately 3×10^{-7} M), well within physiological limits. Any steroid, when given in large enough quantities, will act as an indiscriminate cellular poison, and the tissue culture fibroblast will respond with diminished growth and then death to high concentrations (25 to 100 μg/ml or approximately 10^{-4} M) of even the most inactive anti-inflammatory steroid (4).

Although the direct relationship that exists between fibroblast cell division and inflammation is not understood at this time, there is a correlation of steroid effects in these two processes.

Finally, the steroid biotransformation demonstrated in the presence of these cells (22, 25) and their potential as precursors of many other cell types combined with their ability to perform many of their *in vivo* functions *in vitro*, make this system a highly flexible and potentially unlimited source of information concerning the mechanism of action of anti-inflammatory steroids.

SUMMARY

Fibroblasts in a tissue culture system closely mimic, in their reaction to various corticosteroids, the response of inflammed tissue *in vivo*. When concentrations of steroids in the physiological range (from 0.0001 to 10 μg/ml of medium) are added to strain L fibroblasts, these cells respond with decreased growth and morphological changes similar to those exhibited by connective tissue fibroblasts under corticosteroid influence. Details of the *in vitro* assay method are presented, together with information concerning the statistical treatment of the data obtained. Relative potencies of various naturally occurring and synthetic steroids compared to cortisol (potency = 1), determined in this assay system, range from 0.48 for corticosterone, 1.7 for prednisolone, 11.3 for paramethasone, 7.5 for dexamethasone, 156 for triamcinolone acetonide, and 440 for fluocinolone acetonide. These figures correlate well with the relative activities of these steroids found in clinical trials in topical inflammatory conditions.

REFERENCES

1. Dougherty, T. F. and Schneebeli, G. L.: The use of steroids as anti-inflammatory agents. Ann. N. Y. Acad. Sci. *61:* 328, 1955.
2. Gerarde, H. W. and Jones, M.: The effect of cortisone on collagen synthesis *in vitro*. J. Biol. Chem. *201:* 553, 1953.
3. Castor, W. C. and Baker, B. L.: The local action of adrenocortical steroids on epidermis and connective tissue of the skin. Endocrinology *47:* 234, 1950.
4. Ruhmann, A. G. and Berliner, D. L.: Effect of steroids on growth of mouse fibroblasts *in vitro*. Endocrinology *76:* 916, 1965.
5. Schlagel, C. A.: Comparative efficacy of topical anti-inflammatory corticosteroids. J. Pharm. Sci. *54:* 335, 1965.
6. Berliner, D. L. and Ruhmann, A. G.: Comparison of the growth of fibroblasts under the influence of 11β-hydroxy and 11-keto corticosteroids. Endocrinology *78:* 373, 1966.
7. Dougherty, T. F.: Role of anti-inflammatory adrenal cortical hormones in stress. Transactions Pacific Coast Oto-Ophthalmological Soc., p. 1, 1958.
8. Dougherty, T. F., Berliner, M. L., and Berliner, D. L.: Hormonal control of lymphocyte production and destruction. Progr. Hemat. *3:* 155, 1962.
9. Hollander, J. L., Brown, E. M., Jessar, R. A. and Brown, C. Y.: Hydrocortisone and cortisone injected into arthritic joints. Comparative effects of and use of hydrocortisone a local antiarthritic agent. J. A. M. A. *147:* 1629, 1951.
10. Dougherty, T. F.: Role of Steroids in Regulation of Inflammation. In Mills, L. G. and J. H. Mayer (eds.), Inflammation and Diseases of Connective Tissue, p. 449, W. B. Saunders Co., Philadelphia, Pa.
11. Castor, C. W. and Muirden, K. D.: Collagen formation in monolayer cultures of human fibroblasts. The effect of hydrocortisone. Lab. Invest. *13:* 560, 1964.
12. Ozello, L.: Effects of 17β-estradiol and of growth hormone on the production of acid mucopolysaccharides by human embryonal fibroblasts *in vitro*. J. Cell Biol. *21:* 283, 1964.
13. Berliner, D. L., Ruhmann, A. G., and Berliner, M. L.: The fibroblast—a sensitive target cell for steroids. Excerpta Medica *111:* 189, 1966.
14. Flandre, O., Simonin, L., and Alymeras, M. T.: Action de la beta-methasone sur des fibroblastes en culture *in vitro*. Compt. Rend. Soc. Biol. *158:* 1696, 1964.
15. Grosser, B. I. and Swim, H. E.: *In vitro* response of cultured uterine fibroblasts to

various steroids. Proc. Amer. Assoc. Cancer Res. *18:* 304, 1958.
16. Berliner, D. L., Gallegos, A. J., and Schneebeli, G. L.: Early morphological changes produced by anti-inflammatory steroids on tissue culture fibroblasts. J. Invest. Derm. *48:* 44, 1967.
17. Eagle, H.: Nutrition needs of mammalian cells in tissue culture. Science *122:* 501, 1955.
18. Goldstein, A.: Biostatistics, New York and London, MacMillan Co., p. 122, 1964.
19. Emmens, C. W.: Principles of Biological Assay. London, Chapman and Hall Ltd., 1948.
20. Snedecor, G. N.: Statistical Methods. Ames, Iowa State University Press, 1956.
21. Bush, I. E.: The 11-oxygen function in steroid metabolism. Experientia *12:* 325, 1956.
22. Berliner, D. L. and Dougherty, T. F.: Hepatic an extrahepatic regulation of corticosteroids. Pharmacol. Rev. *13:* 329, 1961.
23. Scher, R. K.: Treatment of refractory dermatoses with fluocinolone acetonide. Curr. Ther. Res. *3:* 461, 1961.
24. Sawyer, W. C.: Treatment of resistant eczematous dermatoses with a new compound, fluocinolone acetonide. Ann. Allergy *20:* 330, 1962.
25. Berliner, D. L.: Biotransformation of corticosteroids as related to inflammation. Ann. N. Y. Acad. Sci. *116:* 1078, 1964.